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Original article

Epstein-Barr virus and breast cancer: Epidemiological and Molecular study on Egyptian and Iraqi women

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KEYWORDS

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Abstract *Background and purpose:* The role of Epstein-Barr virus (EBV) in breast carcinogenesis is still controversial. Unraveling this relationship is potentially important for better understanding of breast cancer etiology, early detection and possibly prevention of breast cancer. The aim of the current study is to unravel the association between EBV and primary invasive breast cancer (PIBC) in two different Arab populations (Egyptian and Iraqi women).

Patients and Methods: The study was done on paraffin-embedded tissues of 40 Egyptian and 50 Iraqi patients with PIBC in addition to 20 normal breast tissues as controls for each group. Both controls and neoplastic tissues were assessed for the expression of EBV genes and proteins (EBNA-1, LMP-1, and EBER) as well as CD21 marker by immunohistochemistry (IHC), in situ hybridization (ISH) and PCR techniques.

Results: Our gold standard for EBV reactivity in breast cancer cases was positivity of both EBNA1 by PCR and EBER by in situ hybridization. EBV was detected in 18/40 (45%) and 14/50 (28%) of Egyptian and Iraqi women; respectively where $p = 0.073$, compared to 0/20 (0%) of their control groups ($p < 0.05$). Regarding the association between EBV positivity and tumor grade, there was not any statistical significant difference between EBV presence and tumor grade in both populations

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where $p = 0.860$ and $p = 0.976$ and the calculated rank biserial correlation coefficient was 0.114 and 0.269 for Egyptian and Iraqi women respectively.

Conclusion: Our findings show that EBV might act as a promoter for the development of PIBC and it might contribute to increased tumor aggressiveness in Egyptian and Iraqi patients.

Introduction

Breast cancer is the most frequently diagnosed malignancy of women in many populations [1]. In Egypt, breast cancer ranked first among all tumors presented to the National Cancer Institute (NCI), Cairo, where it represents 17.5% of all diagnosed cancer cases [2]. Researches into breast cancer etiology have focused primarily on reproductive and other factors affecting circulating sex hormones and on genetic susceptibility. However, as identified risk factors are thought to explain the incidence in about half of all breast cancer cases only, researchers are motivated to consider other routes for disease pathogenesis [3–6]. Viruses have been implicated in the development of various cancers, but they have not been much considered for breast cancer. Identification of the mouse mammary tumor virus (MMTV) supports a viral etiology for breast tumors in animals, though similar germ line viral sequences found in humans are not believed to play any direct role in carcinogenesis [7]. Detection of Epstein-Barr virus (EBV) in the neoplastic tissues of breast cancer cases has been reported by some authors [8–13]. The relationship between breast cancer and EBV could be of potential importance not only for better understanding of breast cancer etiology, but also for early detection, prevention of breast cancer and treatment. A possible association of EBV with breast cancer was proposed as a consequence of the high incidence of male breast cancers, which was reported in Mediterranean countries, an area endemic for EBV; also, the occurrence of some EBV-associated lymphomas in the breast, and the morphological similarities between medullary carcinoma of the breast and nasopharyngeal carcinoma (NPC) [14]. EBV sequences were found in breast tissues and milk, and transfection of p31 fragment of EBV DNA has been shown to immortalize epithelial cells including mammary epithelial cells [1]. Furthermore, it has been reported that breast cancer cells are heterogeneous in terms of EBV genome content and distribution and this raises the possibility that even though EBV might have no etiological role, it can still contribute to tumor development [14].

Most of the studies performed to date to assess the association between EBV infection and breast carcinoma have been done in Western countries, however, consistent results have to be demonstrated from studies conducted all over the world in order to establish a causal relationship. So far, few studies have been reported from Asian countries [13,15,16] and from Egypt [17–19]. In the current study, a combined approach has been applied to overcome the limitation of commonly used methods for EBV detection in breast cancer, which have lymphocytic infiltrates. Each patient was screened for EBNA1 presence by PCR followed by EBER by in situ hybridization (ISH) to localize the EBV and then LMP1gene was amplified by PCR. Also, CD21 detection by IHC was done in order to localize and target EBV receptors. Therefore, our study was conducted to determine the possible relation between EBV infection and primary invasive breast carcinoma (PIBC) cases in two different Arab populations; Egyptian and Iraqi patients; also, to assess the geographic variability as seen in other malignancies associated with EBV.

Patients and methods

Patients and tissue specimens

This study was done on 40 Egyptian and 50 Iraqi patients with PIBC, in addition to 20 normal breast tissue samples from each country as a control group. All patients were newly diagnosed and none of them had a previous history of breast carcinoma or any other malignancy. Paraffin blocks of breast tissues were obtained from the Pathology Departments of the National Cancer Institute, Cairo University, Egypt, from January 2010 to September 2010 and at the same period, from Baghdad Medical College, Baghdad University, Iraq. For each tumor block, five (4μ thick sections) were cut onto positive-charged slides for routine histopathological examination, immunohistochemistry (IHC) and in situ hybridization (ISH). Another five (10μ thick sections) were cut into a sterile Eppendorf tube for subsequent DNA extraction. A written consent was obtained from all patients prior to enrollment in the study, and the ethical committees of the NCI and Baghdad University approved the protocol, which was in accordance with the ethical guidelines of the 2008 Declaration of Helsinki.

EBER in situ hybridization (ISH)

In situ hybridization for the detection of EBV-specific RNAs (EBER) was performed using kits from Dako, Glostrup, Denmark. Samples were analyzed by microscopy for EBER-stained nuclei. Sections from EBV-positive nasopharyngeal carcinoma were used as positive controls.

Immunohistochemistry

The standard streptavidirin-biotin-peroxidase detection technique was performed as previously described [20] using the mouse monoclonal antibody for CD21 (CD21 A-3, Santa Cruz biotechnology, California, USA) against EBV membrane receptor and LMP-1 (Santa Cruz biotechnology, California, USA). The antigen retrieval method was performed by microwave pretreatment in 0.01 M citrate buffer (pH 6.0). Manufacturer's protocols were followed for all procedures. The primary antibody was applied and incubated overnight at 4 °C in a humidified chamber and after 3 washes in PBS, the secondary antibody and the avidin–biotin complex (ABC) were applied to slides. Diaminobenzidine (DAB) was used as a chromogen and sections were counterstained using Mayer's hematoxylin. To evaluate the specificity of the antibodies, known positive and negative tissues were used as controls. Assessment of

CD21 was based on a membranous or membrano-cytoplasmic pattern. Cases were considered positive with >10% stained cells.

DNA extraction

High molecular weight DNA was extracted from the paraffin sections according to standard protocols. Briefly, samples were de-waxed twice with 1 mL xylene for 10 min, washed twice with 100% ethanol, dried and re-suspended in 500 µl PK buffer [50 mmol/L KCl, 10 mmol/LTris–HCl (pH8.3), 2.5 mmol/L MgCl2, 100 µg/mL gelatin, 0.45% Tween20] and 100 µg/mL proteinase K. Samples were digested overnight at 55 °C, extracted with phenol: chloroform: isoamyl, ethanol precipitated. Nucleic acid was dissolved in 100 µL TE storage buffer.

Polymerase chain reaction (PCR)

Tumor and normal DNA were used for PCR, which was done to detect EBNA-1 and LMP-1 expression as previously described by Hashimoto et al. [21]. PCR products were electrophoresed in 2% ethidium bromide-stained agarose gel and visualized with UV light transilluminator (Fig. 1).

Statistical analysis

Statistical analyses were carried out using a program of statistical package for social sciences (SPSS) version 18 (PASW IBM Corp., USA, 2010). Numerical data (age) were presented as mean ± standard deviation (SD) or median and range. Qualitative variables, such as EBV viral markers' presence and clinicopathological features for Egyptian and Iraqi patients, were expressed by frequencies and percentage. Comparison between age groups for Egyptian and Iraqi cases was done using the *t*-test as appropriate. Differences between Egyptian and Iraqi EBV-Positive Breast Carcinomas with regard to clinicopathological features were examined using either Chi-square test ($\chi 2$) or Fisher's exact test when appropriate. Rank biserial correlation coefficient was calculated for studying the strength of association between ordinal variable (tumor grade) and nominal variable (EBV presence). p-value <0.05 was considered significant.

Results

EBV positivity in breast cancer cases

Our gold standard for EBV positivity in breast cancer cases was the detection of EBNA1 antigen by PCR and EBER1 by in situ-hybridization assay. We performed EBER in situ hybridization on the PCR positive cases to localize the positive signal. PCR-Egyptian positive cases were 23/40(57.5%). Out of those 23 patients, 18(45%) cases were positive for EBER (ISH). From those 18 patients, 12(30%) cases were positive for LMP1 (PCR), as illustrated in Fig. 1. Detection of LMP1 antigen by PCR method can distinguish the latent EBV from non-latent infection that was confirmed by the presence of EBV by immunohistochemistry technique in the breast cancer tissues, as shown in Fig. 2. On the other hand, PCR-Iraqi positive cases were 16/50(32%). Out of those 16 patients, 14(28%) cases were positive for EBER (ISH), and 11(22%) cases were positive for LMP1 (PCR). In other words, 11 Iraqi patients had EBV latent infection. Also, CD21 detection by IHC was done in order to localize and target EBV receptors, where it was found in 22/40(55%) and 16/50(32%) of Egyptian and Iraqi patients, respectively. Table 1 shows the frequency of different EBV markers by each technique (LMP1 and EBNA1 by PCR or EBER by ISH, or CD21 by IHC) in the Egyptian and Iraqi groups in addition to their controls. The highest frequency was reported using EBNA-1 PCR assay, where it was positive in 57.5% Egyptian patients. However, the lowest prevalence was reported using LMP1 by PCR assay in 22% of Iraqi patients.

Our results demonstrated that EBV positivity in PIBC tissues was (45%) and (28%) in Egyptian and Iraqi patients respectively (Table 2), while it was absent in the control group 0/20(0%) of both populations. There was a highly statistically significant difference between the prevalence of EBV in tumor and normal tissue samples in both populations ($p = 0.0001$ and $p = 0.005$ for Egyptian and Iraqi cases, respectively).

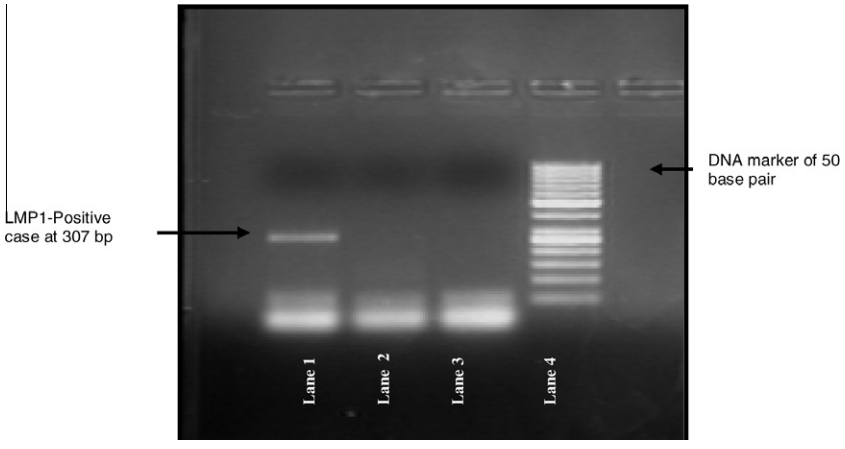

Figure 1 Data on gene amplification. Ethidium bromide-stained 2% agarose gel (Lane 1) showed positive amplification of LMP1 gene in breast cancer case. Lane 2 showed negative amplification of LMP1 gene in breast cancer case. Lane 3 showed Negative-PCR control; Lane 4 showed molecular weight marker.

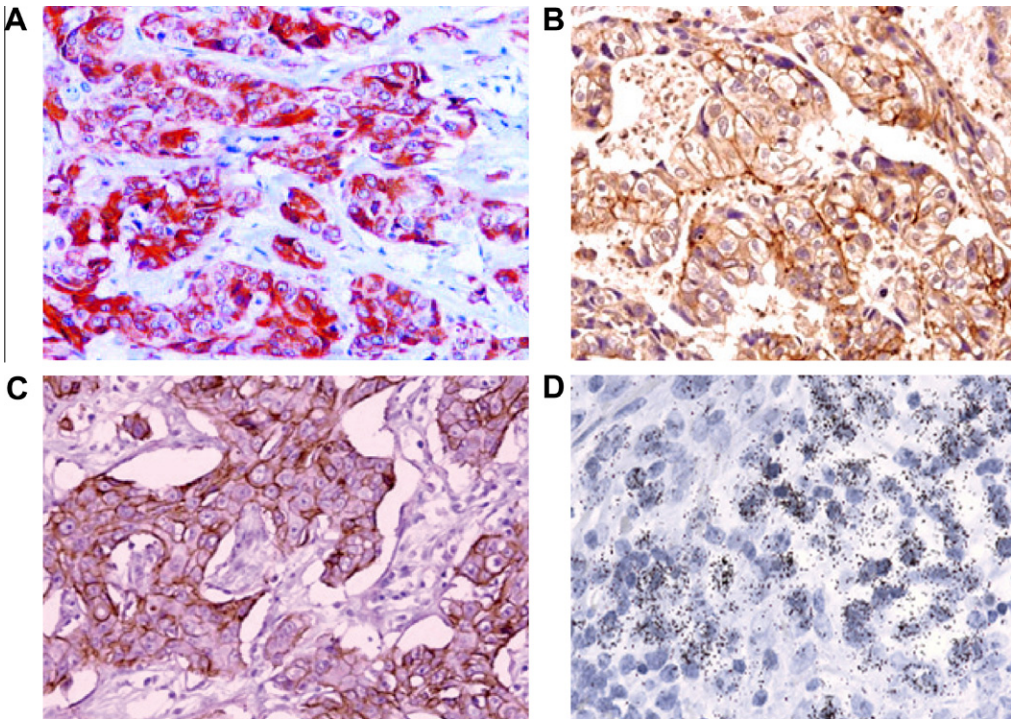

Figure 2 Legend: (A) A case of invasive duct carcinoma showing positive cytoplasmic immunostaining for LMP-1, (B&C) A case of invasive duct carcinoma showing positive membranous immunostaining for CD21, (D) A case of invasive duct carcinoma showing positive nuclear in situ hybridization with EBER-1.

Table 1 Frequency of EBER1, EBNA1, LMP1, and CD21 viral markers in Egyptian and Iraqi EBV-positive breast carcinomas, detected by; in situ hybridization, immunohistochemistry, or PCR technique.

Parameter	No.	EBER1 (ISH)%	EBNA1 (PCR)%	LMP1 (PCR)%	CD21 (IHC)%
Egyptian patients	40	18 (45%)	23 (57.5%)	12 (30%)	22 (55%)
Controls	20	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Iraqi patients	50	14 (28%)	16 (32%)	11 (22%)	16 (32%)
Controls	20	0 (0%)	0 (0%)	0 (0%)	1 (5%)

EBER: EBV-encoded small nonpolyadenylated RNA, EBNA-1: EBV nuclear antigen-1, LMP-1: latent membrane antigen-1, PCR: polymerase chain reaction.

Table 2 Clinicopathological features of Egyptian and Iraqi EBV-Positive Breast Carcinomas, detected by both EBNA1 (PCR) and EBER1(ISH) techniques.

Features	Egyptian patients (+)ve cases%	p-value	Iraqi patients (+)ve cases%	p-value
Age (years)				
< 30– < 50	11/23 (47.8)	0.46	12/41 (29.2)	0.51
50–⩽80	7/17 (41.1)		2/9 (22.2)	
Histology				
Ductal	14/32 (43.8)	0.53	11/42 (26.2)	0.40
Lobular	4/8 (50)		3/8 (37.5)	
Tumor grade				
I	½ (50)	0.93	2/9 (22.2)	0.79
II	13/28 (46.4)		10/32 (31.2)	
III	4/10 (40)		2/9 (22.2)	
Total	18/40 (45)		14/50 (28)	

Table 3 Some previously published studies which evaluated EBV infection in breast cancer specimens by different assays.

Authors, year	Country	Patients No.	Tumor type	Assay	EBV positivity
Luqmani and Shousha (1995) [8]	England	28	Invasive carcinoma, various	Nested PCR for BamH1W ISH for EBER IHC for LMP1	54% 0% Focal positive
Labrecque et al. (1995) [9]	England	91	Invasive carcinoma, various	PCR for BamHI-W	21%
				PCR for EBERs	17/34 (50%)
		19		ISH for BamH1W DNA ISH for EBER1 RNA	12/19 PCR + 6/19 (32%)
Chu et al. (1998) [10]	Taiwan	60	Invasive ductal carcinoma Some with medullary features	IHC for EBNA2	0%
				IHC for LMP1 ISH for EBER1	0% 0%
Glaser et al. (1998) [11]	US	107	Invasive carcinoma, various	ISH for EBER1	0%
Bonnet et al. (1999) [12]	France	100	Invasive ductal, lobular other carcinomas	PCR for EBER2, BZLF1, LMP2	51%
				ISH for EBER1 Southern blot for BamH1W IHC for EBNA1	0/3 PCR + 7/7 (100%) 9/9 (100%)PCR +
Brink et al. (2000) [35]	Holland	24	Carcinoma	PCR for BamH1W PCR for LMP1 rtPCR for BamH1A RNA rtPCR for EBNA1 RNA IHC for EBNA1 ISH for EBER1/2	21% 2/5 (40%) PCR + 0/5 PCR + 0/5 PCR + 1/5 (20%) PCR + 0/5 PCR +
McCall et al. (2001) [45]	US	115	Intraductal, invasive	Microdissection and PCR for EBNA1 ISH for EBER 1/2 IHC for LMP1	2/115 (2%) (50%) PCR + 1/2 PCR +
Dadmanesh et al. (2001) [43]	Italy	4	Lymphoepithelioma-like Carcinoma	ISH for EBER1	0%
Kijima et al. (2001) [44]	Japan	61	Carcinoma	ISH for EBER1	0%
Fina et al. (2001) [36]	North Africa, Europe	509	Invasive ductal carcinoma	PCR for EBER ISH for EBER1 10/20 Microdissection and RT PCR for EBER1	32% (50%) PCR + 2/2 (100%)
Chu et al. (2001) [46]	US	48	Invasive carcinoma	IHC for EBNA1 IHC for LMP1 IHC for BZLF1 ISH for EBER1 PCR for EBNA-4 (EBNA-3b) PCR for LMP1 Southern blot for EBV clonality	25% 0% 0% 10% 10% 10% 0/6 PCR +
Grinstein et al. (2002) [37]	US	33	Infiltrating ductal and lobular carcinoma	IHC for EBNA1 PCR for EBER	42% 14/14 (100%)
Herrmann and Niedobitek (2003) [47]	Germany	59	Invasive ductal, lobular, medullary and undifferentiated carcinoma	PCR for BamH1 W DNA ISH for EBER1 RNA ISH for BamH1 W IHC for EBNA1	7% 0% 0% 0%
Murray et al. (2003) [48]	UK	153	Infiltrating ductal, carcinoma in situ, medullary, and mucinous	RT PCR for Pol Microdissection ISH for EBER IHC for EBNA1	19/92 (21%) 0/19 PCR + 0/19 PCR + 31%

Table 3 Some previously published studies which evaluated EBV infection in breast cancer specimens by different assays.

Authors, year	Country	Patients No.	Tumor type	Assay	EBV positivity
Preciado (2003) [38]	North and South America	102	Carcinoma	IHC for EBNA1 PCR for BamH1W and EBER	38/102 (37%) 24/69 (35%)
Kalkan et al. (2005) [49]	Turkey	57	invasive ductular, lobular, and Other miscellaneous carcinomas	PCR for EBVDNA	13/57(23%)
Perkins et al. (2006) [39]	USA	24	invasive breast carcinoma	real time PCR for EBVDNA	11/24(46%)
Mohamed et al. (2007) [18]	Egypt	34	invasive breast carcinoma	IHC for LMP-1 PCR for EBVDNA	6/34(17.6%) 12/34(35.3%)
Fawzy et al. (2008) [19]	Egypt	40	invasive breast carcinoma	IHC for EBNA-1 PCR for EBVDNA	10/40(25%) 8/40(20%)
Joshi et al. (2009) [13]	India	58	invasive ductular, lobular, and Other malignant carcinomas	IHC for EBNA-1	28/51(54.9%)
Lorenzetti et al. (2010) [40]	Argentine	71	invasive carcinoma	IHC for LMP2A, IHC for EBNA1 IHC for LMP1 ISH for EBER PCR for EBV DNA	16/71(22.5%) 22/71(31%) 0/71(0%) 0/71(0%) 22/71(31%)
EBNA1	23/40(57.5%)		40 Egyptian cases	invasive breast carcinoma ISH for EBER1 PCR for LMP1 IHC for CD21	PCR for 18/40(45%) 12/40(30%) 22/40(55%)
Current study (2011)	Egypt				
		50 Iraqi cases	invasive breast carcinoma	PCR for EBNA1 ISH for EBER1 PCR for LMP1 IHC for CD21	16/50(32%) 14/50(28%) 11/50(22%) 16/50(32%)

Although EBV detection was markedly increased in Egyptian (45%) than Iraqi patients (28%), but this difference was statistically insignificant ($p = 0.073$).

Clinicopathological findings

Egyptian patients' age ranged from 27 to 76 years with a mean age of 48.48 ± 11.16 years, while Iraqi patients' age ranged from 27 to 63 years with a mean age of 42.18 ± 9.06 years. Invasive duct carcinoma (IDC) constituted 80% (32/40) of the Egyptian cases and 84% (42/50) of the Iraqi cases, whereas invasive lobular carcinoma (ILC) constituted 20% (8/40) of the Egyptian cases and 16% (8/50) of the Iraqi cases. In the Egyptian group, 2 cases (5%) were grade I, 28 (70%) were grade II, and 10 (25%) were grade III while, 9 out of the 50 Iraqi cases (18%) were grade I, 32 (64%) were grade II, and 9 (18%) were grade III (Table 2).

Relationship of EBV reactivity with pathological features

Regarding the association between EBV positivity and tumor grade, there was not any statistical significant difference between EBV presence and tumor grade in both populations where $p = 0.860$ and $p = 0.976$, and the calculated rank biserial correlation coefficient was 0.114 and 0.269 for Egyptian and Iraqi women respectively.

Discussion

EBV was classified as a group-1 carcinogen by IARC Working Group [22]. However, the associated cancers vary markedly in viral prevalence, from nearly 100% of nasopharyngeal carcinoma (NPCs) to about 10% of gastric carcinomas [23–25], and also differ in the patterns of viral genes expressed, suggesting that EBV may affect cell growth in more than one way [25]. Thus, EBV infection represents an important but not a sufficient step in carcinogenesis, and epidemiological risk factors have been shown to play an additional critical role in this process.

Indirect support for an association of EBV with breast cancer comes from observations that: (a) EBV is present in breast tissue, where it is detected in breast milk in some women [26]; (b) transfection of EBV DNA stimulates growth of human breast milk cells [27]; (c) some EBV-associated lymphomas occur in the breast [28,29]; (d) breast cancer has epidemiological similarities to young-adult Hodgkin's lymphoma, although evidence for breast cancer implicates timing of primary EBV infection rather than viral oncogenesis [30]; (e) EBV has been identified in benign breast tumors in immunosuppressed women [31]; (f) in vitro, breast epithelial cells can be infected by direct contact with EBV-bearing lymphoblastoid cell lines [32]; and (g) Regarding serological evidence, measurement levels of anti-EBNA-1 Immunoglobulin (IgG) antibodies in stored sera of Breast Cancer Indian Women was previously

done by Joshi et al. [13] using commercial Enzyme linked Immunosorbent Assay (ELISA) kit, in which patients with benign breast diseases were used as a comparison group for both immunohistochemical and serological analysis.

In the current study, multiple analytical assays have been used for evaluation of different EBV gene products under study. First assay was In situ Hybridization for EBER detection where it enables us for differentiating EBV in tumor cells from EBV in surrounding lymphocytes. Second and third assays were PCR for amplification of EBNA-1 and LMP-1 detection where PCR is potentially a highly sensitive and specific method but it cannot differentiate EBV in malignant breast epithelial cells from EBV in surrounding lymphocytes. The fourth assay was Immunohistochemistry for CD21 detection in order to localize and target EBV receptors.

Our results have shown that EBV positivity in PIBC tissues was (45%) and (28%) in Egyptian and Iraqi patients respectively. This observation may be explained by the fact that EBV DNA presence in breast cancer patients can differ between groups with different demographic distributions and population characteristics, as stated by Wiencke [33]. In other words, distribution of HLAs may differ in Egyptian population than in Iraqi population. However, because of the numbers of samples tested, these observations should be confirmed in population-based studies involving larger numbers of samples.

In addition, EBV detection was absent in the control group of both populations ($p < 0.05$), where the normal breast tissues were obtained from different normal subjects. Although most of the previous studies have used normal breast tissues distant to tumor, we preferred to assess EBV in normal breast tissues obtained from reduction mammoplasty specimens for two reasons. First, several previous studies on different solid tumor types provided evidence that what is called adjacent normal tissues is only morphologically normal since many molecular changes have been detected in these areas. Second, in most breast cancer cases the adjacent normal tissues contain many inflammatory cells and lymphocytes which are the normal reservoir for EBV and this could affect the final results [34–35].

This finding is in close to the previous study done by Joshi et al. [13] where they found that about 55% of breast cancer Indian women cases showed EBNA-1 expression in tumor cells by IHC, while all the controls with benign breast disease were negative. Also, two Egyptian authors Mohamed et al. [18] and Fawzy et al. [19] have reported EBV infection in 35.3% and 25% of invasive breast carcinoma respectively; however, those three studies had certain limitations, where they looked for expression of only one viral protein. They should have investigated multiple EBV proteins present in different phases of viral latency seen in other EBV associated tumors. Also, our findings are close to numerous studies done by Horiuchi et al. [36], Luqmani and Shousha [8], Labrecque et al. [9], Bonnet et al. [12], Brink et al. [37], Fina et al. [38], Grinstein et al. [39], Xue et al. [27], Preciado, [40], Perkins et al. [41], and Lorenzetti et al. [42], where EBV infection has been found most consistently and in a prevalence of about 10–50% using PCR method, and histochemical methods have produced evidence of viral DNA or proteins within the breast cancer cells that suggests a pathogenic role of EBV. Although EBV infection has been found by several authors, it has been completely undetected by others such as Gaffey et al. [43], Lespagnard et al. [44], Glaser et al. [11], Dadmanesh et al. [45], and Kijima et al. [46], as illustrated in Table 3. The inconsistency and apparent variability of these findings should not itself exclude a role of EBV in breast carcinogenesis because it is influenced to some extent by three issues: First, the marked variation in EBV prevalence even among studies using the similar techniques as the studies done by Bonnet et al. [12], Brink et al. [37], and Deshpande et al. [51]; Second, the possibility of false-positive or -negative analytical test results due to lymphocyte-derived EBV, cross-reactivity immunostains, amplicon contamination, or inappropriate technique sensitivity; and the last issue is the absence of EBV-cancer association hallmarks including EBERs expression in all cells of virus-associated tumors. Breast cancer may express EBERs less abundantly than other malignancies [9–12]. However, EBERs are not found in all NPCs or in some Burkitt and Hodgkin lymphomas that are LMP-1 positive by PCR [11–23].

Regarding the association of EBV presence with tumor grade, there was no statistical significant relation between EBV presence and tumor grade ($p > 0.05$) in both populations. The correlation coefficient indicates a weak correlation where it was 0.114 and 0.269 for Egyptian and Iraqi patients respectively. This finding disagrees with that done by Mazouni et al. [50] where they stated that the proportion of EBV-positive samples increased significantly with increasing tumor grade, from 16.2% for grade I, to 32.0% and 46.4% for grades II and III, respectively. The inconsistency in both results might be due to; (a) the small sample size where our study was done on 40 Egyptian and 50 Iraqi patients compared to 196 French breast tumor cases, and (b) the various laboratory methods that were applied in our study for assessment of EBV genes and proteins (immunohistochemistry (IHC), in situ hybridization (ISH) and PCR), while real time PCR assay has been used by Mazouni et al. [52] for EBV detection.

In conclusion, our findings suggest that EBV might have a role in the pathogenesis of breast cancer. These findings were based mainly on EBV detection by PCR technique and confirmed by immunohistochemistry and in situ hybridization techniques in breast cancer patients from different geographic region. So, EBV may contribute to increased tumor aggression even in patients from different geographic region.

Further research is necessary in order to determine the role of EBV in the etiology or progression of breast cancer, particularly; it was detected in the lymphocytes around the breast tissues suggesting its contribution as a modulating agent in the microenvironment area around those breast tissues. In other words, it may modulate the secretion of interleukin-6 and tumor necrosis factor which have proven to have a role in breast cancer development.

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References

[1] Glaser SL, Hsu JL, Gulley ML. Epstein-Barr virus and breast cancer: state of the evidence for viral carcinogenesis. Cancer Epidemiol Biomarkers Prev 2004;13:688–97.

[2] El Saghir NS, Khalil MK, Eid T, El Kinge A-R, Charafeddine M, Geara F, Seoud M, Shamseddine AI. Trends in epidemiology and management of breast cancer in developing Arab countries: a literature and registry analysis. Int J Surgery 2007;5(4):225–33.

[3] Hulka BS, Moorman PG. Breast cancer: hormones and other risk factors. Maturitas 2001;38:103–13.
[4] Martin AM, Weber BL. Genetic and hormonal risk factors in breast cancer. J Natl Cancer Inst 2000;92:1126–35.
[5] Madigan MP, Ziegler RG, Benichou J. Proportion of breast cancer cases in the United States explained by well-established risk factors. J Natl Cancer Inst 1995;87:1681–5.
[6] Mezzetti M, La Vecchia C, Decarli A, Boyle P, Talamini R, Franceschi S. Population attributable risk for breast cancer: diet, nutrition, and physical exercise. J Natl Cancer Inst 1998;90(389–94):11.
[7] Serraino D, Piselli P, Scognamiglio P. Viral infections and cancer: epidemiological aspects. J Biol Regul Homeost Agents 2001;15:224–8.
[8] Luqmani YA, Shousha S. Presence of Epstein-Barr virus in breast carcinoma. Int J Oncol 1995;6:899–903.
[9] Labrecque LG, Barnes DM, Fentiman IS, Griffin BE. Epstein-Barr virus in epithelial cell tumors: a breast cancer study. Cancer Res 1995;55:39–45.
[10] Chu JS, Chen CC, Chang KJ. In situ detection of Epstein-Barr virus in breast cancer. Cancer Lett 1998;124:53–7.
[11] Glaser SL, Ambinder RF, DiGiuseppe JA, Horn-Ross PL, Hsu JL. Absence of Epstein-Barr virus EBER-1 transcripts in an epidemiologically diverse group of breast cancers. Int J Cancer 1998;75:555–8.
[12] Bonnet M, Guinebretiere JM, Kremmer E, et al. Detection of Epstein-Barr virus in invasive breast cancers. J Natl Cancer Inst 1999;91:1376–81.
[13] Joshi D, Quadri M, Gangane N, Joshi R, Gangane N. Association of Epstein Barr Virus Infection (EBV) with Breast Cancer in Rural Indian Women. Plos One 2009;4(12):e8180.
[14] Mant C, Hodgson S, Hobday R, D'Arrigo C, Cason J. A viral aetiology for breast cancer: time to re-examine the postulate. Intervirology 2004;47:2–13.
[15] He JR, Tang LY, Yu DD, Su FX, Song EW, Lin Y, Wang SM, Lai GC, Chen WQ, Ren ZF. Epstein-Barr virus and breast cancer: serological study in a high-incidence area of nasopharyngeal carcinoma. Cancer Lett 2011;309(2):128–36.
[16] Perrigoue JG, den Boon JA, Friedl A, Newton MA, Ahlquist P, Sugden B. Lack of association between EBV and Breast Carcinoma. Cancer Epidemiol, Biomarkers Preven 2012;1(4).
[17] Bahnassy AA, Zekri A-R N, Saad N A, El-Houssini S, Khalid HM, Sedky LM, Mokhtar NM. Epstein–Barr viral infection in extranodal lymphoma of the head and neck: correlation with prognosis and response to treatment. Histopathology 2006;48:516–28.
[18] Mohamed WS, Mohamed MA, Omar MM. Possible involvement of Epstein-barr Virus (EBV) in pathogenesis and prognosis of female breast infiltrating duct carcinoma: clinicopathological, immunohistochemical and molecular study. The Egy J Med Microbiol 2007;16(2):403–14.
[19] Fawzy S, Sallam M, Awad NM. Detection of Epstein-Barr virus in breast carcinoma in Egyptian women. Clin Biochem 2008;41(7-8):486–92, Epub 2008 Jan 3.
[20] Pan L, Diss TC, Peng H, Lu Q, Wotherspoon AC, Thomas JA, Isaacson PG. Epstein-Barr virus (EBV) in enteropathy-associated T-cell lymphoma (EATL). J Pathol 1993;170:137–43.
[21] Hashimoto Y, Nawata Y, Kurasawa K, et al. Investigation of EB virus and cytomegalovirus in rapidly progressive interstitial pneumonitis in polymyositis/dermatomyositis by in situ hybridization and polymerase chain reaction. Clin Immunol Immunopathol 1995;77:298–306.
[22] IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Epstein - Barr virus and Kaposi's sarcoma Herpesvirus/Human Herpesvirus8. Lyon:IARC;1997.
[23] Hsu JL, Glaser SL. Epstein-Barr virus-associated malignancies: epidemiologic patterns and etiologic implications. Crit Rev Hematol Oncol 2000;34:27–53.
[24] Gulley ML. Review Molecular diagnosis of Epstein-Barr virus-related diseases. J Mol Diagn 2001;3(1):1–10.
[25] Niedobitek G, Meru N, Delecluse HJ. Epstein-Barr virus infection and human malignancies. Int J Exp Pathol 2001;82:149–70.
[26] Junker AK, Thomas EE, Radcliffe A, Forsyth RB, Davidson AG, Rymo L. Epstein-Barr virus shedding in breast milk. Am J Med Sci 1991;302:220–3.
[27] Xue SA, Lampert IA, Haldane JS, Bridger JE, Griffin BE. Epstein-Barr virus gene expression in human breast cancer: protagonist or passenger? Br J Cancer 2003;89:113–9.
[28] Giardini R, Piccolo C, Rilke F. Primary non-Hodgkin's lymphomas of the female breast. Cancer 1992;69:725–35.
[29] Abhyankar SH, Chiang KY, McGuirk JP, et al. Late onset Epstein-Barr virus-associated lymphoproliferative disease after allogeneic bone marrow transplant presenting as breast masses. Bone Marrow Transplant 1998;21:295–7.
[30] Yasui Y, Potter JD, Stanford JL, et al. Breast cancer risk and "delayed" primary Epstein-Barr virus infection. Cancer Epidemiol Biomark Prev 2001;10:9–16.
[31] Kleer CG, Tseng MD, Gutsch DE, et al. Detection of Epstein-Barr virus in rapidly growing fibroadenomas of the breast in immunosuppressed hosts. Mod Pathol 2002;15:759–64.
[32] Speck P, Longnecker R. Infection of breast epithelial cells with Epstein-Barr virus via cell-to-cell contact. J Natl Cancer Inst 2000;92:1849–51.
[33] Wiencke JK. Impact of race/ethnicity on molecular pathways in human cancer. Nat Rev Cancer 2004;4:79–84.
[34] Zhou X, Meeker KA, Makambi HK, Kosti O, Kallakury VSB, Mary K Sidawy KM, Loffredo CA, Zheng YL. Telomere length variation in normal epithelial cells adjacent to tumor: potential biomarker for breast cancer local recurrence. Carcinogenesis 2011;33(1):113–8.
[35] Wels J, Kaplan NR, Rafii S, Lyden D. Migratory neighbors and distant invaders: tumor-associated niche cells. Genes Dev 2008;22:559–74.
[36] Horiuchi K, Mishima K, Ohsawa M, Aozasa K. Carcinoma of stomach and breast with lymphoid stroma: localisation of Epstein-Barr virus. J Clin Pathol 1994;47:538–40.
[37] Brink AATP, van den Brule AJC, van Diest P, Meijer CJLM. Re: detection of Epstein-Barr virus in invasive breast cancers. J Natl Cancer Inst 2000;92:655–6.
[38] Fina F, Romain S, Ouafik L, et al. Frequency and genome load of Epstein-Barr virus in 509 breast cancers from different geographical areas. Br J Cancer 2001;84:783–90.
[39] Grinstein S, Preciado MV, Gattuso P, et al. Demonstration of Epstein-Barr virus in carcinomas of various sites. Cancer Res 2002;62:4876–8.
[40] Preciado MV. Lack of evidence for an association of Epstein-Barr virus infection with breast carcinoma-another point of view. Breast Cancer Res 2003;5:E6.
[41] Perkins RS, Sahm K, Marando C, Dickson-Witmer D, Pahnke GR, Mitchel M, Petrelli NJ, Berkowitz IM, Soteropoulos P, Aris VM, Dunn SP, Krueger LJ. Analysis of Epstein-Barr virus reservoirs in paired blood and breast cancer primary biopsy specimens by real time PCR. Breast Cancer Res 2006;8:R70.
[42] Lorenzetti MA, De Matteo E, Gass H, Martinez Vazquez P, Lara J, Gonzalez P, Preciado MV, Chabay PA. Characterization of Epstein Barr virus latency pattern in Argentine breast carcinoma. PLoS One 2010;5(10):e13603.
[43] Gaffey MJ, Frierson Jr HF, Mills SE, et al. Medullary carcinoma of the breast. Identification of lymphocyte subpopulations and their significance. Mod Pathol 1993;6:721–8.
[44] Lespagnard L, Cochaux P, Larsimont D, Degeyter M, Velu T, Heimann R. Absence of Epstein-Barr virus in medullary carcinoma of the breast as demonstrated by immunophenotyping, in situ hybridization and polymerase chain reaction. Am J Clin Pathol 1995;103:449–52.

[45] Dadmanesh F, Peterse JL, Sapino A, Fonelli A, Eusebi V. Lymphoepithelioma-like carcinoma of the breast: lack of evidence of Epstein-Barr virus infection. Histopathology 2001;38:54–61.

[46] Kijima Y, Hokita S, Takao S, et al. Epstein-Barr virus involvement is mainly restricted to lymphoepithelial type of gastric carcinoma among various epithelial neoplasms. J Med Virol 2001;64:513–8.

[47] McCall SA, Lichy JH, Bijwaard KE, Aguilera NS, Chu WS, Taubenberger JK. Epstein-Barr virus detection in ductal carcinoma of the breast. J Natl Cancer Inst 2001;93:148–50.

[48] Chu PG, Chang KL, Chen YY, Chen WG, Weiss LM. No significant association of Epstein-Barr virus infection with invasive breast carcinoma. Am J Pathol 2001;159:571–8.

[49] Herrmann K, Niedobitek G. Lack of evidence for an association of Epstein-Barr virus infection with breast carcinoma. Breast Cancer Res 2003;5:R13–7.

[50] Murray PG, Lissauer D, Junying J, et al. Reactivity with a monoclonal antibody to Epstein-Barr virus (EBV) nuclear antigen 1 defines a subset of aggressive breast cancers in the absence of the EBV genome. Cancer Res 2003;63:2338–43.

[51] Deshpande CG, Badve S, Kidwai N, Longnecker R. Lack of expression of the Epstein-Barr Virus (EBV) gene products, EBERs, EBNA1, LMP1, and LMP2A, in breast cancer cells. Lab Invest 2002;82:1193–9.

[52] Mazouni C, Fina F, Romain S, Ouafik L, Bonnier P, Brandone J-M, Martin P-M. Epstein-Barr virus as a marker of biological aggressiveness in breast cancer. Br J Cancer 2010;10:1038.